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Thymus allograft biopsies were performed in athymic infants with complete DiGeorge anomaly after thymus transplantation to assess whether the thymus allograft tissue was able to support thymopoiesis. Forty-four consecutive infants were treated with postnatal cultured thymus allografts. Thirty biopsies and six autopsies evaluating the allograft site were obtained in 33 infants, 23 of whom survive. The allograft was examined by immunohistochemistry for evidence of thymopoiesis. Grafted thymus tissue was found in 25 of 30 biopsies, 23 of which showed thymopoiesis. All 19 survivors with thymopoiesis on biopsy developed naive T cells and T cell function. Autopsies were done in six subjects, three of whom had biopsies. All autopsy samples contained thymus tissue including one for which the biopsy had not contained graft. Of the six autopsies, one had evidence of thymopoiesis. Epithelium without thymopoiesis was seen in two of 25 biopsies in which thymus tissue was detected and in five of six autopsies. Graft rejection was seen in one autopsy. Biopsies were important for showing the following: 1) the damaging effect of pulse steroids on thymopoiesis; 2) the need for adequate immunosuppression of atypical subjects; and 3) the presence of thymopoiesis in the presence of ongoing immunosuppression. In addition, the biopsy could rule out graft rejection in the atypical subjects who had oligoclonal T cells that could cause rejection. In summary, combining biopsy and autopsy data, allogeneic thymus tissues showed thymopoiesis in 24 of 29 (86%) evaluable transplants. The results of these biopsies led to improved care of these complex patients. The Journal of Immunology, 2008, 180: 6354–6364.

DiGeorge anomaly is a congenital anomaly in which the heart, parathyroid, and thymus are commonly affected (1–4). Less than 1% of the patients appear to be athymic; these patients are classified as having complete DiGeorge anomaly (5). Complete DiGeorge anomaly is a fatal condition. Based on 15 historical controls (Ref. 5 and our unpublished observations), two-thirds of children with complete DiGeorge anomaly can be expected to die by 1 year and the rest by ~2 years of age.

Athymia in the context of DiGeorge anomaly is defined as those infants having the following: 1) <50 naive T cells/mm³; or 2) naive T cells comprising <5% of total T cells (6). Naive T cells are T cells that have recently emerged from the thymus. They coexpress the markers CD45RA and CD62L (7). Athymia cannot be accurately diagnosed by examination of a chest radiograph or computerized tomography scan or even by the visual inspection of the mediastium at surgery. Blood testing for naive cell markers is critical for the diagnosis of athymia.

Complete DiGeorge anomaly has two distinct clinical presentations. Athymic patients with very few T cells and no rash have typical complete DiGeorge anomaly. However athymic infants often develop oligoclonal T cells over time that are associated with rash, lymphadenopathy, and, in some cases, hepatomegaly (8). These T cells do not express CD45RA and CD62L. These athymic infants are classified as having developed the phenotype of atypical complete DiGeorge anomaly. Biopsies of affected skin and liver show T cell infiltration. Infiltrating T cells may be CD4 single positive or CD8 single positive or may be double negative (CD4−CD8−). The rashes can be very severe. The rashes associated with atypical complete DiGeorge anomaly clinically resemble those in Omenn syndrome (9), immunodeficiency/polyendocrinopathy/enteritis/X-linked syndrome (IPEX) (10), engraftment of maternal T cells (11), or graft vs host disease (GVHD). To rule out GVHD or maternal engraftment, it is necessary to isolate circulating T cells and prove that they are host and not maternal or third party. The T cell numbers in patients with atypical complete DiGeorge anomaly can increase to very high levels, even >40,000/mm³.

This report focuses on what was learned through the immunohistochemical evaluation of biopsies of 30 thymus allografts and autopsy evaluation of six grafts. When graft biopsies showed rejection or lack of thymopoiesis, pretransplantation therapy was altered resulting in engraftment of subsequent subjects. When autoimmune outcomes developed posttransplantation, the biopsy provided information to determine whether there had been technical problems with the graft. In part because of changes instituted based on biopsy and autopsy results, the results of transplantation of thymus tissue into infants with typical or atypical DiGeorge

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4 Abbreviations used in this paper: GVHD, graft vs host disease; CK, cytokeratin; RSV, respiratory syncytial virus; TCRBV, TCR β-chain variable region.

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anomaly has improved. Survival, naive T cell development, and T cell function are found in ~70% of the infants. Of the 44 infants transplanted, 31 survive with a median followup of 4.7 years.

Materials and Methods

Patient populations

All infants with complete DiGeorge anomaly transplanted with thymus tissue from 1993 through December 2006 are included in this report. All subjects and thymus donors were enrolled in protocols approved by the Duke Institutional Review Board (Durham, NC). Subsequent to 2001, all protocols were also reviewed by the Food and Drug Administration and conducted under an investigational new drug application. The parent(s) of research subjects and thymus donors provided informed consent. In all cases, of the 44 transplants, graft biopsies were performed in 30. Biopsies were not performed in the remaining 14 infants due to infection in three (respiratory syncytial virus (RSV), parainfluenza virus, and CMV), unrepaired heart defects or cardiac risk factors in four, tracheostomy or pulmonary instability in three (one of whom also had cardiac instability and pulmonary bacterial infections), and death before the anticipated time of biopsy in four. Twenty-three healthy adults enrolled under an Institutional Review Board-approved protocol provided blood as controls for TCR Vbeta chain variable region (TCRBV) analysis by flow cytometry. Six patients undergoing heart surgery were enrolled as potential thymus donors under an Institutional Review Board-approved protocol. Their parents provided informed consent. The blood of these infants was evaluated by flow cytometry for TCRBV3 expression.

The immunosuppression protocol used in 22 subjects has been described (6). Approximately one-third of the transplanted subjects (14/44) had atypical complete DiGeorge anomaly characterized by rash, lymphadenopathy, and oligoclonal T cells. This group of subjects required immunosuppression. The immunosuppression in the initial subject with atypical complete DiGeorge syndrome was deoxycoformycin only. Most of the other atypical subjects received three doses of pretransplantation rabbit anti-thymocyte globulin. Two of these subjects had additional immunosuppression as described previously (6).

Thymus transplantation and biopsy

Thymus transplantation was performed as described (6, 12, 13). In brief, thymectomy or partial thymectomy is frequently performed by pediatric cardiothoracic surgeons to gain access to the surgical field. That thymus tissue is discarded. The discarded thymus tissue was collected and informed consent was obtained from the donor’s parent(s) before the use of the thymus tissue for transplantation. The thymus tissue was sliced and held in culture for 12–21 days before transplantation (14). Thymus slices were inserted into the quadriceps muscle in an open procedure in the operating room. Sutures were placed at the surface of the muscle to indicate the implantation sites. The muscle was irrigated with sterile saline. An incision was made through the scar over one quadriceps. The muscle bed was exposed. Biopsies were obtained under four marking sutures. It was not possible to grossly identify thymus material.

Immunohistochemistry

Biopsies of the thymus allograft were usually conducted at 2–3 mo posttransplantation if the medical condition of the subject was stable (6, 15, 16). The biopsy in one subject was delayed because of the medical condition of the subject but was then done at 7 mo when surgical placement of a central line was required. Frozen and paraffin-embedded sections were reacted with a panel of Abs including cytokeratin (CK) (clones AE1/AE3; Dako) CD3 (polyclonal; Dako), CD1a (clone 010; Beckman Coulter), Ki-67 (clone mib-1; Beckman Coulter) (15, 16), and CK10 (clone DE-K10; NeoMarkers, Lab Vision). Cytokeratin was used to identify thymus tissue in the tissue samples. CD1a and Ki-67 were used to identify cortical thymocytes. For TCRBV3 staining, clone A2 from Immunotech (Coulter) was used. This Ab reacts with the molecule commonly called TCRBV3. TCRBV3 has been renamed TCRBV28 in the international ImmunogeneTics database (imgt.cines.fr:8104). TCRBV28 is a single gene family. Control tissue was stained at the time of every subject assay to assure that all Abs were working appropriately. Histologic evidence of thymopoiesis in biopsies was defined as the presence of a lacy pattern of CD3-positive thymic epithelial cells and the presence of CD3+CD1a+Ki-67+ cells (cortical thymocytes).

Other immune evaluations

Standard flow cytometry was done as previously reported (6, 15).

Results

Table I presents a summary of the biopsy data from our study. The data are stratified by presenting clinical and immune phenotype (typical vs atypical) and by the type of immunosuppression that was used. Sutures were left by the transplant surgeon to mark the site of thymus tissue in the quadriceps muscles. Approximately four tissue samples were obtained during the biopsy. The presence of CK in the biopsy specimen confirmed that the graft had been sampled. If there was no CK, then the graft site had been missed. As expected, the survival of the subjects with thymopoiesis was better than the survival of all subjects because some subjects died shortly after transplantation and did not undergo a biopsy. All survivors developed naive T cells by 1 year except for one child who did not undergo a biopsy due to a heart condition.

The presence of Hassall bodies was not required for the definition of thymopoiesis. The biopsies without Hassall bodies appeared to consist of cortex only, although CK14, a marker of medullary thymic epithelium, was invariably present. As all thymus grafts had Hassall bodies at the time of transplantation and because only 60% of the biopsies had Hassall bodies, we assumed that Hassall bodies were resorbed after transplantation and only developed in situ if cortex and medulla became distinct areas.

We were curious as to whether subjects with biopsies that had large areas of thymopoiesis with many thymocytes would yield higher naive CD4 counts at 1 year vs subjects who had biopsies with few or no thymocytes. These biopsy categories compared subjects from all treatment groups. There were 17 subjects with biopsies showing large areas with many cortical thymocytes. Of these 17 subjects, 16 survived past one year. Naive T cell counts were done at 1 year in 15 subjects. (The first subject was transplanted before the development of Abs to recognize naive T cells.) The average naive CD4 count at 1 year for the 15 evaluable subjects with large areas of thymopoiesis characterized by lacy CK and cortical thymocytes was 279/mm3. There were two subjects having biopsies with CK that did not show thymopoiesis and six subjects with biopsies that showed thymopoiesis but had few cortical thymocytes. Of these eight subjects, four survived to 1 year. The average naive CD4 count for the four evaluable subjects with very few thymocytes on biopsy was 178/mm3. The difference in the naive CD4 T cell counts at 1 year did not reach significance (p = 0.07; one-tailed t test).

The allograft site was evaluated at autopsy in three subjects who did not undergo a biopsy. In all three samples CK was present, including in two atypical subjects and one typical subject. Thymopoiesis was only seen in the typical subject. A fourth subject died on the day of transplantation from causes unrelated to the transplantation and the allograft site was not examined.

Subject safety at time of biopsy

To ensure the safety of these research subjects, biopsies were not performed if the subjects had serious pulmonary, infectious, or cardiac problems. Thus, biopsies were not done in 14 of the 44 transplant recipients (five of whom died before the usual time of biopsy). If possible, allograft biopsies were scheduled to coincide with other necessary surgical procedures. With this policy in place, no serious adverse side effects to graft biopsies were observed.
Table I. Thymus allograft biopsies

<table>
<thead>
<tr>
<th>Phenoype/Treatment</th>
<th>Typical, No</th>
<th>Typical, RAGAM</th>
<th>Typical, CsA</th>
<th>Atypical, RATGAM</th>
<th>Atypical, Deoxycoformycin</th>
<th>Atypical, CsA + Other</th>
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</thead>
<tbody>
<tr>
<td>Biopsy Results</td>
<td></td>
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<tr>
<td>Suppression</td>
<td></td>
<td></td>
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<tr>
<td>RATGAM</td>
<td>47/7</td>
<td>4/4</td>
<td>12/16</td>
<td>4/4</td>
<td>9/11</td>
<td>11/12 (92%)</td>
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<tr>
<td>Subtotal</td>
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<td>0/0</td>
<td>0/0</td>
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<td>0/0</td>
</tr>
<tr>
<td>Total</td>
<td>167/22</td>
<td>4/4</td>
<td>12/16</td>
<td>4/4</td>
<td>9/11</td>
<td>11/12 (92%)</td>
</tr>
</tbody>
</table>

No. of biopsies/total subjects: 16/22, 4/7, 20/29, 4/5, 4/4, 2/2, 0/1, 10/15, 30/44.

CK with thymopoiesis/total biopsies: 12/16, 3/4, 15/20, 4/4, 3/4, 1/2, 0/0, 8/10, 23/30.

CK but no thymopoiesis/total biopsies: 0/16, 0/3, 8/15, 3/4, 0/3, 1/1, 0/0, 2/8, 10/23.

Large areas with many cortical thymocytes/no. with thymopoiesis: 10/12, 3/3, 13/15, 1/4, 3/3, 0/1, 0/0, 4/8, 17/23.

No. of surviving/subjects with biopsy: 17/22 (77%), 6/7 (86%), 23/29 (79%), 4/5 (80%), 5/7 (71%), 0/2 (0%), 0/1 (0%), 9/15 (60%), 32/44 (73%).

**Abbreviations:** CK, cytokeratin; CsA, cyclosporine; HB, Hassall bodies; RATGAM, rabbit anti-thymocyte gamma globulin.

**DIG003 also had autopsy tissue with CK but without thymopoiesis, no HB.**

**DIG004 also had autopsy tissue with CK, but no thymopoiesis, no HB.**

**DIG104 also had autopsy tissue with CK but no thymopoiesis, no HB.**

Thymopoiesis was found in 23 biopsies

Fig. 1 shows a representative biopsy from subject DIG024 who presented with typical complete DiGeorge anomaly and who did not receive immunosuppression. The biopsy shows evidence of thymopoiesis with corticomedullary distinction, lacy CK, cortical thymocytes reactive with CD1a and Ki-67 Abs, and medulla with CK10⁺ Hassall bodies. The lower left area of each panel in Fig. 1 is a cortical area with thymocytes expressing Ki-67 and CD1a. The medullary area in the center of each panel has a Hassall body and some associated CD1a⁺ dendritic cells. In D there are a few CD1a⁺ cortical thymocytes near the Hassall body; although at low power the medullary and cortical areas are distinct. Biopsies in 22 other subjects were similar in showing lacy CK and cortical thymocytes. These biopsies are classified as having thymopoiesis in Table I. Ten of the 23 biopsies with thymopoiesis had Hassall bodies, including the one shown in Fig. 1. We believe that the 13 biopsies showing cortical thymocytes but no Hassall bodies represented thymus cortex or thymus before the development of a distinct medullary area.

There were six biopsies of the 23 with thymopoiesis that did not have large areas containing cortical thymocytes. Two of these were in typical subjects. These biopsies had only one or two small areas of thymus tissue, but these areas had densely packed cortical thymocytes. The other four biopsies were in atypical subjects. In these four biopsies there were only scattered cortical thymocytes in the area of CK. Three of these four had not been treated with cyclosporine. In general, the subjects with the biopsies having fewer cortical thymocytes had been sicker prior to biopsy.

Early in the course of this research as we were first encountering subjects with atypical complete DiGeorge anomaly, the finding of thymopoiesis on biopsy let us know that we had made the correct diagnosis and that athymia was responsible for the lack of naive T cells and the presence of an oligoclonal repertoire. If the subjects had instead an autoimmune disease with an intact thymus, the transplanted thymus would have been rejected. Likewise, as atypical complete DiGeorge anomaly patients resemble those with Omenn syndrome (9), finding thymopoiesis in those early atypical subjects confirmed their athymia because Omenn syndrome patients need bone marrow, not thymus, to develop normal T cells (17). The early biopsy findings were instrumental in confirming the criteria for the diagnosis of atypical complete DiGeorge anomaly and for our continued efforts in transplantation in this group of patients.

Biopsies in five subjects in whom thymus tissue was not identified

No CK-positive material was identified in the biopsy tissue in five of the 30 total biopsies obtained. These tissue samples contained either fat or muscle, with no evidence of surgical changes in the sections examined. It was concluded that these biopsies did not encompass the surgical site. It was noted that tissue samples that contained predominately fat tended to float when placed into the formalin fixative. Therefore, after the first seven biopsies the biopsy procedure was modified to obtain four tissue samples. Half of each sample was placed in formalin to determine whether it would sink or float to avoid analysis of fat tissue. This minimized the possibility of future noninformative biopsies.

Effect of immunosuppression on thymus function

A subject with typical complete DiGeorge anomaly, DIG002, died on day 66 posttransplantation after four days of therapy with 5
mg/kg/day hydrocortisone for an intraventricular bleed. The autopsy revealed the presence of a Hassall body, indicating that thymopoiesis had recently been occurring in the tissue, although no T cells were found in the biopsy. DIG002 developed 129/mm³ phenotypically normal T cells on day 49 after transplantation and did not have a rash. The appearance of these T cells reflected thymopoiesis in the graft. The lack of thymocytes on biopsy likely reflected depletion secondary to 4 days of steroid therapy. At the time it was unknown whether thymopoiesis would have recurred after this course of high-dose steroids if the subject had survived the intracranial hemorrhage.

Subject DIG003 presented with typical complete DiGeorge syndrome but developed respiratory failure associated with T cells in the blood on day 35 posttransplantation. The subject was treated with 40 mg/kg/day methylprednisolone for 3 days and the respiratory condition markedly improved. In retrospect we believe that these T cells were oligoclonal, similar to what is seen in atypical complete DiGeorge anomaly. These oligoclonal amplifications can occur any time before the development of naive T cells. A biopsy was attempted on day 111 posttransplantation, but no CK-positive material was identified. The subject died on day 130 from sepsis with no evidence of T cells having developed. On autopsy, CK was detected at the site of the transplant but thymopoiesis was not detected in the sections examined (Fig. 2, A and B). Rejection was ruled out as there were no T cells infiltrating the graft. The pulse steroid therapy on day 35 posttransplantation likely permanently damaged the thymus tissue, preventing subsequent thymopoiesis. We concluded from DIG003 that steroid pulses with doses as in DIG003 could irreversibly damage the thymus. The question arose as to whether immunosuppression with cyclosporine, lower levels of steroids (e.g., 2 mg/kg/day methylprednisolone for limited periods of time), and rabbit anti-thymocyte globulin would damage the thymus. Fig. 2, C–F, shows a biopsy from a subject treated with cyclosporine from 44 days before transplantation through the biopsy on day 70 after transplantation. Trough levels were mainly 150–220 ng/ml during this time. DIG204 had weaned prednisolone from 2 mg/kg/day immediately after transplantation to 1 mg every other day (weight, 6.8 kg) at the time of transplantation. The biopsy showed excellent thymopoiesis. Note the presence of thymocytes expressing both Ki-67 and CD1a (Fig. 2, D and E),

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**FIGURE 1.** Thymopoiesis in a graft recipient (DIG024) at 91 days posttransplantation. Frozen sections of the allograft biopsies were stained with H&E (A) and reacted with Abs to pan-CK (B), CK10 (C), CD1a (D), CD3 (E), and Ki-67 (F). CK10 reacts specifically with Hassall Bodies. CD1a and the nuclear proliferation marker Ki-67 are proteins characteristically found in cortical thymocytes. All panels are at original magnifications of ×40; the bar in A is 50 μm.

**FIGURE 2.** Effect of immunosuppression on subsequent graft function. Immunohistochemistry with CK Ab on formalin-fixed tissue obtained from the autopsy of DIG003, who was treated with pulse steroids (A and B). Condensed thymic epithelium is seen without associated lymphocytes. A, Original magnification is ×10; bar is 100 μm. B, Original magnification is ×40; bar is 50 μm. Biopsy is of the allograft in DIG204, who was treated with cyclosporine and steroids (C–F). Reactivity is with CK (C), Ki-67 (D), CD1a (E), and CD3 (F).
which are markers characteristic of cortical thymocytes. We concluded that cyclosporine and steroids in these moderate doses did not prevent thymopoiesis.

Variability of biopsy samples

As discussed in Materials and Methods, the thymus tissue is sliced before culture and the slices are implanted in the quadriceps at transplantation. There is variability in the appearance of biopsy depending on the slice examined. This likely reflects the initial slicing of the thymus before culture. Some slices may contain predominantly a fibrous area, whereas others are cortex. Normally, four small pieces of tissue are obtained at the time of biopsy, and each is divided in half. Half of each piece of tissue is analyzed by frozen section and half is embedded in paraffin and then examined. In Fig. 3 is an example of two biopsy samples from the same subject having disparate appearances, with thymopoiesis detected in one tissue sample but not in the other. The area shown in Fig. 3, A–C, has lacy CK (A) along with cortical thymocytes expressing CD1a (B) and Ki-67 (C). The area shown in Fig. 3, D–F, shows condensed CK (D) without any associated cortical thymocytes (E and F). Allograft biopsies are considered positive for thymopoiesis if there are any areas of lacy CK with cortical thymocytes in any tissue samples derived from that subject. The biopsy in Fig. 3, therefore, was characterized as having thymopoiesis. These data show that a biopsy without thymopoiesis may merely reflect a sampling artifact.

Presence of CK but lack of thymopoiesis in two graft biopsies and one graft on autopsy; association with GVHD, infection, and insufficient immunosuppression

In two of 25 subjects in whom graft was identified on biopsy and in one of the four subjects whose grafts were evaluated by autopsy but not biopsy, CK was present but the graft did not appear to be functioning in thymopoiesis. DIG111 presented with preexisting CMV infection and GVHD from a blood transfusion. Immunosuppression of DIG111 included pretransplantation cyclophosphamide and rabbit anti-thymocyte globulin and pre- and posttransplantation cyclosporine and steroids. The biopsy obtained on day 65 after transplantation. The CK was condensed and there were no cortical thymocytes (Fig. 4, A and B). The T cells seen in Fig. 4B did not express Ki-67 or CD1a (data not shown). These T cells, in fact, may represent some of the T cells (from the unirradiated blood transfusions) that may have damaged the thymus graft as well as caused cutaneous GVHD and other symptoms in the subject. DIG111 died from these pretransplantation conditions despite use of pretransplantation cyclophosphamide and rabbit anti-thymocyte globulin and pre and posttransplantation treatment with cyclosporine and steroids. Presumably the graft was damaged from the CMV, GVHD, or drugs such as ganciclovir used for treatment. No evidence of CMV-infected cells was found by immunostaining (data not shown).

DIG401 was an atypical subject who presented with CMV infection. DIG401 was treated with ganciclovir before transplantation and foscarnet for the first month after transplantation, after which ganciclovir was reinstituted. Cytomegalovirus immune globulin intravenous (Cytogam) and/or intravenous Ig (IVIG) were used throughout his course. The CMV viral load was low at the time of transplantation but rose a month later leading to profound brain damage, blindness, and death 103 days after transplantation. Cytokeratin was identified in autopsy specimens but no thymopoiesis was found (Fig. 4, C and D). It is likely that CMV infection and/or ganciclovir treatment is a risk factor for graft failure. As in DIG111, however, no evidence of CMV-infected cells was found on immunostaining of this sample (data not shown).

DIG113 was a subject whose biopsy specimen, although containing CK, did not appear to be functioning. This subject presented with atypical complete DiGeorge anomaly with a severe rash and predominantly CD8 oligoclonal T cells. DIG113 was treated with pretransplantation rabbit anti-thymocyte globulin and steroids. All suppression was stopped on the day of transplantation. On day 13 after transplantation, the subject’s rash had returned, the liver had enlarged, the bilirubin had increased, and the T cell count had increased to 11,993/mm³. At that point cyclosporine and steroids were started. A biopsy was done on day 77. The CK was very condensed (Fig. 4E) and there were very few associated T cells (Fig. 4F) none of which expressed Abs for CK (A and D), CD3 (B and E), and Ki-67 (C and F). All figures have an original magnification of ×20; the bar in D is 50 μm.
Ki-67 (not shown). There was no evidence of graft rejection. The conclusion was that the graft was not functioning. The subject later developed a normal CD4:CD8 ratio (day 167) and a normal T cell repertoire. Naive T cell numbers remained lower than in other subjects, only reaching 99/mm³ by day 602. Thus, the graft at the time of the biopsy still had the potential for thymopoiesis but the poor appearance on biopsy correlates with the poorer outcome in terms of naive T cell count. This subject provided the rationale for using more suppression in atypical subjects. In fact, all subsequent atypical subjects have been treated with peritransplant cyclosporine and steroids. It is possible that the poor appearance of this tissue may have reflected sampling variability. No other subjects who had a biopsy without thymopoiesis progressed to develop naive T cells.

An important lesson learned from DIG113 was to not retransplant immediately based on lack of thymopoiesis in the biopsy. We had planned to repeat a thymus transplant in DIG113 based on lack of thymopoiesis. Shortly before we planned to perform a second transplantation 4 mo after the biopsy, naive T cells appeared in the blood. This example illustrates the limited negative predictive value of thymus allograft biopsies that have little thymopoiesis. In future instances of biopsies not showing thymopoiesis, we will follow naive T cell percentages for an additional 4–5 mo before deciding that the graft has not functioned.

Different appearance of biopsies in subjects receiving portions of the same cultured thymus

Biopsy histology was also compared in situations in which two recipients received thymus allografts from the same donor. In the first case, both subjects received pretransplant rabbit antithymocyte thymoglobulin. Atypical complete DiGeorge anomaly subject DIG106 received the allogeneic thymus tissue transplant after 16 days of culture, and typical complete DiGeorge anomaly subject DIG105 received it after 21 days of culture. Fig. 5, A and B, show that the graft biopsy in DIG105 (done on day 78 after transplantation) has good thymopoiesis with many cortical thymocytes and lacy CK. The graft in DIG106 (done on day 118 after transplantation) has few cortical thymocytes and more condensed CK (Fig. 5, C and D). However, in both subjects the thymocytes expressed CD1a and Ki-67, which are markers of cortical thymocytes (not shown). Thus, both biopsies were classified as having thymopoiesis. Of interest, DIG106 had been critically ill in the peritransplantation period and was on a ventilator for 1 month beginning 8 days before transplantation. It is possible that the stress of the poor medical condition of DIG106 led to the slow development of thymopoiesis in this graft and poorer function of the thymus with time. The development of naive CD4 T cells in DIG106 occurred later compared with the development of these cells in DIG105. On day 161 DIG105 had 76 naive CD4 T cells/mm³ whereas DIG106 only had 6/mm³ on day 159. At 1 year both subjects had similar numbers of naive CD4 T cells, with DIG105 having 262/mm³ and DIG106 having 288/mm³ on days 372 and 368, respectively. However, at 4.24 years DIG105 had 305/mm³ naive CD4 T cells, whereas DIG106 had 162/mm³ at 4.9 years. Thus, DIG105, who had more thymopoiesis on biopsy, has higher naive CD4 T cells after 4 years. We acknowledge that it is possible that the difference in the appearance relates strictly to sampling of the biopsy.

Graft rejection

Graft rejection was seen in one subject in an autopsy specimen. This subject (DIG101) was the first subject to present with atypical complete DiGeorge anomaly and was described previously (8). DIG101 received only two doses of deoxycoformycin before transplantation. Stronger immunosuppression was not used because of medical instability. The subject died of respiratory failure on day 44 after transplantation. The diagnosis of graft rejection was based on the condensed CK (Fig. 6, A and B) surrounded by a dense infiltrate of CD3⁺ T cells (Fig. 6, C and D). Those T cells did not express CD1a, which characterizes cortical thymocytes (data not shown). After it was found that this graft had been rejected, all subsequent subjects needing immunosuppression were given rabbit anti-thymocyte globulin before transplantation (6). In contrast to our caution in interpretation of grafts without thymopoiesis, this tissue sample clearly showed rejection. If this had been found on biopsy we would have recommended immediate transplantation with increased immunosuppression.
A subject whose graft initially functioned but then stopped functioning

It is likely that severe stress can damage thymus tissue. Subject DIG104 presented with a severe RSV infection and required intubation for respiratory failure from day 49 to day 77 posttransplantation. Methylprednisolone was begun at 2 mg/kg/day on day 56 and was tapered off by day 100. DIG104 had multiple episodes of staphylococcal coagulase negative line infections and yeast urine infections. His graft was biopsied on day 98 when he went to the operating room for central line placement. The biopsy showed thymopoiesis (Fig. 7, A–C) with lacy CK and cortical thymocytes. Methylprednisolone was restarted at 0.5 mg/kg i.v. every 12 h on day 111 as a treatment for infantile spasms (a seizure disorder). The dosage was increased briefly to 1.5 mg/kg/day but was...
stopped because it was ineffective. Adrenocortical hormone was used briefly beginning on day 119 in an unsuccessful attempt to control the seizures. DIG104 had a respiratory arrest on day 120 and died on day 137 after transplantation. At autopsy, the allograft had no evidence of thymopoiesis (Fig. 7, D–F). There was condensed CK without cortical thymocytes. With autopsies, many samples are taken so it is unlikely that thymopoiesis was missed. We concluded that the severity of the RSV and other infections, the prolonged treatment with ribavirin, the stress from the respiratory failure, and the prolonged treatment with steroids adversely affected thymus graft function in this subject. This finding emphasizes the importance in the peri-transplant period of avoiding stress and infections if at all possible.

**Frequency of TCRBV3 expression in graft biopsy**

Reconstitution of the TCR repertoire is a reflection of thymic output during thymopoiesis. The TCRBV repertoire was evaluated by flow cytometry of peripheral blood in all subjects after transplantation and compared with 23 healthy adult controls. Although the percentages of T cells in most TRBV families were similar to those of healthy adult controls within several months after transplantation, several subjects had percentages of TCRBV3 that were significantly different from the adult controls. These subjects had multiple TCRBV3 measures at different time points. By modeling a mixed linear model with repeated measures for the TCRBV3 outcomes, we found that TCRBV3 in the subjects was significantly lower than in the adult control group ($p = 0.0052$). Eight of the subjects with typical complete DiGeorge anomaly had percentages of T cells bearing TCRBV3 in the first years after thymus transplantation that were $<2$ SD below the mean for adult controls (Fig. 8). Ten had percentages above this value. The percentages of T cells bearing TCRBV3 in the subjects were also less than those of six infants under 9 mo of life ($p = 0.0017$, using a generalized estimating equations (GEE) analysis). All of the atypical subjects (not shown) had TCRBV3 expression in the normal range.

It can be seen that some typical subjects continue to have a deficit in TCRBV3 expression for years. This deficit raised the question as to whether there was inappropriate negative selection for this TCRBV family in the thymus.

All available graft biopsies were examined from all subjects with typical complete DiGeorge anomaly who had evaluation of peripheral blood TCRBV repertoire by flow cytometry. This included four subjects who had low percentages of TCRBV3 (DIG019, DIG024, DIG032, and DIG201) and three who had normal percentages (DIG012, DIG026, and DIG031). The data points for these seven subjects are shown in Fig. 8 in the dark lines. All seven donor thymuses used for transplantation in these subjects were examined by immunohistochemistry for the...
presence of thymocytes expressing TCRBV3. Sample immuno-histochemistry data from thymuses given to four subjects are shown in Fig. 9. All thymuses on the day of harvest expressed TCRBV3-bearing T cells (Fig. 9, A, C, E, and G). TCRBV3+ thymocytes were detected in the biopsy specimens of those subjects who expressed TCRBV3 in peripheral blood after transplantation (DIG012 and DIG026) (Fig. 9, F and H). Interestingly, TCRBV3+ thymocytes were not detected in the biopsy specimens from subjects who had low percentages of TCRBV3 in the blood posttransplantation (DIG032 and DIG024) (Fig. 9, B and D). This suggests that the etiology of the low frequency of TCRBV3 bearing cells is not related to the thymus (which supported TCRBV3 expression in the donor) but to the recipient. Negative selection is thought to be effected by APC that migrate to the thymus from the bone marrow. Thus, the defect here may be related to host APC.

Discussion

Presence of thymopoiesis in the allograft

Obtaining biopsies of the thymus allograft following thymus transplantation has been important for several reasons. First, the finding of thymopoiesis indicated that the development of naive T cells was taking place in the donor thymus. If there had been any native thymus making naive T cells, those T cells would have rejected the graft. Documenting thymopoiesis was especially important in the early years of this research when the first infants with atypical complete DiGeorge anomaly were encountered, especially as some of the infants were normal for chromosome 22q11.1. The possibility existed that these infants could have Omenn syndrome (9, 17). Finding thymopoiesis with the development of genetically recipient T cells in a subject whose phenotype was unusual confirmed the initial diagnosis of athymia. The diagnosis could not have been SCID/Omenn syndrome because a thymus epithelial transplant cannot cure SCID/Omenn syndrome; only bone marrow is curative. After diagnosing and treating the initial infants with atypical complete DiGeorge anomaly, we were able to recognize the phenotype with more certainty. The finding of 22q11 hemizygosity in only half of patients with complete DiGeorge anomaly (6, 15, 18) became accepted based on these biopsy findings. Secondly, it was useful to know that the graft was functioning at the time the subject was discharged back to the referring hospital. Because the biopsy was usually done at 2 mo and circulating naive T cells normally developed at 4–5 mo, it was reassuring (to the parents of the research subject and to the investigator) to know early that thymopoiesis was underway.

Effect of immunosuppression on graft development

We evaluated the effect of steroid therapy on biopsy/autopsy appearance. The effect of pulse steroids at high levels (40 mg/kg/d for 3 days of methylprednisolone) early after transplantation as in subject DIG003 appeared to be detrimental. That subject never developed circulating naive T cells and the graft appearance suggested that the epithelium was permanently dedifferentiated. Because of the concern of damage from early use of pulse steroids, our routine use of steroids in the subjects with atypical complete DiGeorge anomaly is limited to 2 mg/kg/day methylprednisolone or prednisolone after transplantation. The use of immunosuppression has not appeared to adversely affect the thymus architecture based on comparisons with biopsies from subjects with typical complete DiGeorge anomaly who have not received any suppression.

We were concerned that use of cyclosporine might adversely affect thymopoiesis. Of the 10 biopsies in subjects with atypical complete DiGeorge anomaly (all of whom were given immunosuppression), four subjects did not receive cyclosporine. Three of these biopsies met only the minimum criteria for thymopoiesis (lacy CK and Ki-67-positive thymocytes). After changing the clinical protocol to include cyclosporine for atypical subjects, graft appearance was improved. In particular, three of four biopsies of infants with atypical complete DiGeorge anomaly treated with cyclosporine (not counting the two subjects with “other” therapies) showed large areas of cortex with many cortical thymocytes (13). The improved appearance of the graft biopsies in atypical subjects on immunosuppression supported the hypothesis that this change would enhance thymopoiesis in this group of subjects.

The autopsy in DIG003 was helpful in one other way. It showed that the lack of T cell development was secondary to a nonfunctional graft. Viable CK was clearly present. This autopsy allowed us to rule out graft rejection and lack of vascularization of the tissue. In either of these cases, CK would not be expected to be present at the graft site. Instead, only fibrosis would be detected.

Graft rejection and graft failure

The thymus allograft biopsies allowed the subjects to be evaluated for graft failure and graft rejection. Changes were made in the

FIGURE 9. TCRBV3 expression in thymus graft biopsies reflects expression in peripheral blood. The donor thymus and biopsy specimens are shown for DIG032 (A and B) and DIG024 (C and D). The donor thymus and biopsy specimens are also shown for DIG012 (E and F) and DIG026 (G and H). The donor thymuses on the day of harvest are in the top row. The biopsy specimens are in the lower row. Original magnification is ×40; bar is 50 μm.
transplant protocols in an attempt to prevent these problems. Because we found graft rejection in the first atypical subject who was only given deoxycoformycin (Fig. 5), subsequent atypical subjects were treated with rabbit antithymocyte globulin before transplantation. As can be seen in Table I, of four biopsies done in infants with atypical complete DiGeorge anomaly treated with pretransplantation rabbit anti-thymocyte globulin alone, only one of the four had robust thymopoiesis (with three having few cortical thymocytes on biopsy). Of the three biopsies done in subjects in whom cyclosporine and steroids were started before the rabbit anti-thymocyte globulin and transplantation, all three biopsies showed robust thymopoiesis as reported for DIG120 (13). The final group of 2 subjects with the most intensive regimen of suppression died; but their deaths were secondary to the complex problems in those subjects, including CMV infection and GVHD from unirradiated blood transfections before thymus transplantation in one and RSV infection in the other. It is clear from the biopsies shown that immunosuppression in the doses used in the current thymus transplantation protocols does not prevent thymopoiesis.

**Evaluation of risk factors**

The biopsy of DIG111 and the autopsy of DIG401 (Fig. 3) suggest that CMV and GVHD or the drugs to treat these conditions are detrimental to the development of thymopoiesis in transplanted thymus tissue. This might be expected because of similar findings in bone marrow transplantation. We have confidence that sufficient tissue has been obtained in the autopsy specimens for us to know whether or not thymopoiesis has occurred. In fact, we resect the entire area of transplantation to be able to section all possible transplant sites. Thus, we remain very concerned about the ability of subjects to overcome CMV infection after thymus transplantation.

In DIG104, thymopoiesis was lost between biopsy and autopsy (Fig. 7). That subject had severe RSV infection and was treated with i.v. ribavirin. It is unclear whether RSV or ribavirin is to blame for the graft failure in DIG104. We treated three other subjects (DIG019 DIG029, and DIG112) with prolonged inhaled and i.v. ribavirin for parainfluenza virus type III, respectively; all developed naive T cells at the expected time. Thus, we feel that ribavirin does not interfere with thymopoiesis in subjects after transplantation.

**Sampling artifacts**

It was important to obtain approximately four small pieces of thymus allograft for microscopic analysis. It was not possible to actually distinguish the thymus allograft from surrounding muscle even in an open biopsy. Thus, the transplant surgeon left sutures on the surface of the quadriceps muscle to indicate where biopsies should be obtained. Each of the four pieces of tissue obtained was divided in half. One half was evaluated by frozen section and the other half was evaluated after formalin fixation and embedding in paraffin. Thymus tissue was often found in only one or two of the four pieces. If thymopoiesis was seen in any section, that biopsy was considered positive for thymopoiesis.

We found differences in the extent of thymopoiesis in different tissue specimens from the same subject and differences in biopsies from subjects receiving tissue from the same thymus. Some areas of CK-positive epithelium appeared nonfunctional, others had only cortex, and other areas had cortex, medulla, and Hassall bodies. This was likely related to the variability of the tissue used for transplantation. Initially the donor thymus was cut into slices ~0.5-mm thick. Transplantation involved placement of 20–80 pieces. Because the entire thymus was sliced, some slices were predominantly interlobular septum, others predominantly cortex, and others, medulla. This led to the variations in graft appearance on biopsy. Sampling artifacts may also be responsible for lack of correlation between appearance of the allograft biopsy and naive T cell counts at 1 year.

**TCRBV3 expression in blood and allograft biopsies**

The finding that TCRBV3 may not develop in parallel with the other TCRBV families is interesting but of unknown significance. The normal values in all six control infants who underwent heart surgery contrast with the finding of low TCRBV3 percentages in approximately half of transplant recipients (Fig. 8) who had prolonged periods with low TCRBV3 percentages. In the subjects with complete DiGeorge anomaly, excessive infections are not seen after transplantation in the group with low levels of TCRBV3.

In summary, biopsy of thymus allografts has proven to be useful in the evaluation of thymus transplantation in infants with complete DiGeorge anomaly. The data from the biopsies confirm the underlying mechanism put forth for thymus transplantation, namely that recipient bone marrow-derived prothymocytes migrate to the donor allograft and develop there into genetically recipient T cells. It is reassuring to learn that thymopoiesis occurs with immunosuppression such as with cyclosporine. It is also helpful to know that high "pulses" of steroid can damage the thymus in the initial months after transplantation. Our future plans to evaluate these biopsies include the assessment of promiscuous expression of tissue-specific Ags in the allografts and the correlation of that with the development of autoimmune disease.

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**Disclosures**

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**References**


